

The Neuroprotective Effect and Possible Mechanism of Picroside II Against Oxygen Glucose Deprivation/Reoxygenation Cell Model

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Abstract Objective: This study aims to delineate whether autophagy plays an critical role in the neuroprotective effects of picroside II in vitro and further explore potential molecular mechanism. Methods: The oxygen glucose deprivation/reoxygenation (OGD/R) cell model were established in SH-SY5Y cells. The cell viability, cells morphologic change and apoptosis were observed by cell counting kit-8 (CCK-8), inverted microscope and flow cytometry respectively. Autophagy-related proteins Beclin 1, microtubule associated protein 1 light chain 3 (LC3), and p62 levels of brain tissues and cells were detected by Western Blot; furthermore, LC3 expression and autophagy flux of cells were further detected by immunofluorescence labeling and GFP-mRFP-LC3 adenovirus transfection. Reactive oxygen species (ROS) levels and phospho-AMPK, phospho-mTOR, phospho-ULK 1 levels were detected using ROS assay Kit, Western blot and immunocytochemistry, respectively. Results: Picroside II down-regulated of autophagy-related Beclin-1 and LC3 levels, and up-regulated of p62 protein; meanwhile ameliorated the abnormal morphological structures of nerve cells and apoptosis; and increased cell viability. Furthermore, accompanied by decreasing autophagy flux, picroside II prevented the generation of ROS, down-regulated of AMPK and the up-regulated of mTOR and Unc-51-like kinase 1 (ULK1) levels. Conclusion: Picroside II exerts neuroprotective effect against OGD/R injury by inhibiting ROS-mediated AMPK-mTOR-ULK1 autophagy signaling pathway

Keywords: autophagy, OGD/R, AMPK-mTOR-ULK 1, signaling pathway

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1. Introduction

Previous studies have demonstrated that autophagy is a ubiquitous intracellular degradation system in eukaryotic organisms, and its role in diseases such as diabetes [1], neurodegenerative diseases [2], and myocardial infarction [3] has been confirmed. Autophagy also exhibited neuroprotective effects via interruption of apoptosis by decomposing the damaged mitochondria and endoplasmic reticulum [4]. Recently, a series of experiments have shown that inhibition of autophagy can alleviate the damage in vitro [5]. Several studies have demonstrated that autophagy may be a potential therapeutic target for the treatment of cerebral ischemia reperfusion injury. Picroside II is the main component of Picrorhiza which has been shown to play an important role in human health including anti-inflammatory [6], anti-oxidant [7], neuroprotective [8,9] and hepatoprotective [10,11].

Picroside II can exert neuroprotective effects by inhibiting reactive oxygen species (ROS) levels in the rat model of cerebral ischemia reperfusion. In vitro studies have shown that picroside II and nerve growth factor (NGF) synergistically scavenged reactive oxygen species (ROS), protecting PC12 cells from oxidative stress damage caused by H₂O₂ [12]. Therefore, picroside II can alleviate cerebral ischemia-reperfusion injury by inhibiting oxidative stress. However, there is no clear study on whether picroside II can alleviate oxidative stress by regulating autophagy and thus alleviate cerebral ischemia-reperfusion injury. In this study, we conducted a series of experiments in vitro models of OGD/R injury, demonstrating that picroside II exerts neuroprotective effects and molecular mechanism by modulating autophagy to alleviate oxidative stress. ROS can play an important role in cell survival, death, and immune defense by regulating multiple signaling pathways [13]. Adenosine monophosphate-activated protein kinase (AMPK) is a major energy metabolism sensor that regulates energy

balance and metabolic stress by controlling various homeostatic mechanisms such as autophagy and protein anabolism. There are increasing indications that AMPK is activated by oxidative stress such as ROS [14]. ROS can potentially activate AMPK [15], while AMPK has been proven to initiate autophagy [16]. When AMPK is activated, it cooperates with mammalian target of rapamycin (mTOR) in conjunction with downstream ULK 1 to regulate autophagy caused by cerebral ischemia [17]. Based on this, we made a hypothesis that ROS-mediated AMPK-mTOR-ULK 1 pathway may be involved in the protective effect of picoside II on autophagy-induced OGD/R injury.

2. Materials and Methods

2.1. Reagents

Picoside II (C23H28O13, 512.48, 4839012-20-9) was purchased from Tianjin Kuqing Med Tech, China. Compound C (Dorsomorphin, HY-13418) and Cell counting kit-8 (HY-K0301) were purchased from MCE USA. DMEM/High glucose medium (SH30243.01) was purchased from Hyclone USA. DMEM no glucose medium (#11966-025) was purchased from Gibco USA. Anti-AMPK Ab (#2603), anti-phosph-AMPK Ab (#2535), anti-mTOR Ab (#2983), anti-phosph-mTOR Ab (#5536), anti-phospho-ULK1 Ab (#14202), anti-ULK1 Ab (#8054), anti-LC3A/B Ab (#4108), anti-SQSTM1/p62 (#39749) and anti-Beclin 1 (#3495) were purchased from Cell Signaling Technology USA. Anti-LC3B Ab (#18725-1-AP) was purchased from Proteintech USA. Donkey anti-rabbit IgG (H&L) CyTM3 AffiniPure fluorescent secondary antibody (#711-165-152) was purchased from Jackson ImmunoResearch USA. Enhanced BCA assay kit (No. P0010) and 4,6-diamidino-2-phenylindole (DAPI, C1005) were purchased from Beyotime Institute of Biotech, China. ROS assay kit (DCFH-DA, CA1410) was purchased from Beijing Solarbio Tech. China. Annexin V-FITC apoptosis detection kit (40302ES50) was purchased from Yeasen China. In situ cell death detection kit (11684795910) was purchased Roche USA. mRFP-GFP-LC3 double-labeled adenovirus (HH20180323GZH-AP01) was purchased from Hanbio Biotech, China.

2.2. Cell Lines and Cell Model

SH-SY5Y cell lines were purchased from Chinese Academy of Sciences Cell Bank. Cells were planted Dulbecco's modified Eagle's medium (DMEM) high glucose supplemented with 10% fetal bovine serum, placed in a 37 °C, 5% CO₂ incubator.

SH-SY5Y cells were plated in 6 well at 5×10⁵ per well for 24 h and established a glucose OGD/R model allowed when cells reached 80%. DMEM high glucose medium containing 10% fetal bovine serum was replaced with glucose-free serum-free DMEM medium, placed in a constant temperature 37 °C incubator, continuously filled with oxygen-free gas (90% N₂, 9% CO₂ and 1% O₂), taken out after 9h, replaced with DMEM high-sugar medium containing 10% fetal bovine serum, and placed in an incubator at 37 °C, 5% CO₂ for 4 h. Cells were divided

into four groups after culture 24 h, as follows: control group, model group, picoside II group and compound C group. The cells of the control group were cultured under normal conditions. The SH-SY5Y cells of model group were treated by OGD/R as described above. The SH-SY5Y cells of picoside II group were treated by OGD, and treated with picoside II (20μmol/L) during the period of re-oxygenation. The SH-SY5Y cells of compound C group were also treated by OGD, and treated with compound C (10μmol/L) during the period of re-oxygenation.

2.3. Cell Counting Kit-8 (CCK-8)

In order to observe the changes in the growth status, quantity and morphology of each group of cells, five non-overlapping views were randomly selected under inverted microscope after OGD/R.

SH-SY5Y cell were plated in 96 wells at 1×10⁴ per well for 24h and established a OGD/R model allowed when cells reached 80%. After the modeling, added 90 μL of normal medium and 10 μL of CCK-8 solution to each well, in 37 °C incubator incubation 3 h, at the same time set a blank well as a control. The 450 nm OD value of each well was measured by microplate reader.

2.4. Flow Cytometry

After establishing the OGD/R model, the culture medium was aspirated, the cells were washed 3 times with PBS, digested and centrifuged and collected softly. Apoptotic cells were analyzed using flow cytometry (BD AccuriTM C6 Plus, USA), after staining 15min with Annexin V (AV)/PI apoptosis kit in the dark.

2.5. ROS Detection

After OGD/R, each group of cells was treated separately according to the above treatment, meanwhile six duplicate holes are set in each group. Intracellular ROS were detected by active oxygen test kit through installing the DCFH-DA ROS fluorescent probe according to the instructions. The microplate reader (BIO-TEK, H1M, USA) with excitation and emission wavelengths of 488 and 525 nm respectively, were used to determine fluorescence intensity. Results were expressed as a percentage of the control group.

2.6. Western Blot Analysis

The cells were deprived of glucose and oxygen for 9 h and re-oxygenated for 4 h, rinsed gently twice with cold PBS and then dissolved in buffer containing RIPA with both phosphatase and protease inhibitors. The cell homogenate from each group was collected into a 1.5 mL Eppendorf tube. After cleavaging on ice for 15 min, the cell homogenate was centrifuged for 15 min at 4 °C, 3600 g in a refrigerated centrifuge (Eppendorf 5801 type, Germany) to remove cellular debris. The protein concentration in the supernatant is detected by the BCA quantitation kit. Total protein of tissues or cells was separated on 15%, 10%, 8% SDS-polyacrylamide gel (Bio-Rad), transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore,

USA). The PVDF membranes were blocked with 5% BSA in TBST, and incubated with the following antibodies 4oC overnight: rabbit anti-LC3A/B (1:1,000); rabbit anti-Beclin 1 (1:1,000); rabbit anti-p62/SQSTM1 (1:1,000); rabbit anti-phospho AMPK (1:1,000), rabbit anti-total AMPK (1:1,000); rabbit anti-phospho mTOR (1:1,000), rabbit anti-total mTOR (1:1,000); rabbit anti-phospho ULK 1 (1:1000); rabbit anti-total AMPK (1:1,000). After washing 3 times with the TBST, and then incubated with the HRP-labeled secondary antibodies (1:5,000) for 1 hours at room temperature. Bands were visualized with ECL method, and analyzed using Image J system. The density of each band was compared with GAPDH (1:5,000) or β -actin (1:5,000). The density of LC3 II was compared with LC3 I.

2.7. Immunocytochemistry

The cells were deprived of glucose and oxygen for 9 h and re-oxygenated for 4 h, and fixed in 4% paraformaldehyde for 15 min, and then rinsed 3 times with PBS for 5min each time. Fixed cells were then blocked with 10% donkey serum containing 0.3% Triton-X for 1 h at room temperature. Cells were then incubated with the following primary antibodies for 2 h at room temperature including rabbit anti-LC3B (1:500), rabbit anti-phospho AMPK (1:300), rabbit anti-phospho mTOR (1:200). After washing 3 times with PBS, cells were incubated with the secondary antibodies for 1h at room temperature: donkey anti-rabbit IgG (H&L) CyTM3 affiniPure (1:800). After rinsing 3 times, it was incubated with DAPI stain for 5min. Immunoreactivity was detected by Nikon fluorescence microscope. Five non-overlapping fields were randomly selected under a fluorescence microscope.

2.8. Virus Transfection

SH-SY5Y cells were plated in 24 wells containing coverslips at 1×10^5 per well and allowed to reach 50%-70% confluence at the time of transfection. Cells were

transfected with mRFP-GFP-LC3 adenoviral vectors containing polybrene (8 μ g/mL). Eight hours later, cells were switched to 10% serum-containing medium for 48h, and used for experiments. Images were captured by confocal microscope. Five non-overlapping fields were randomly selected.

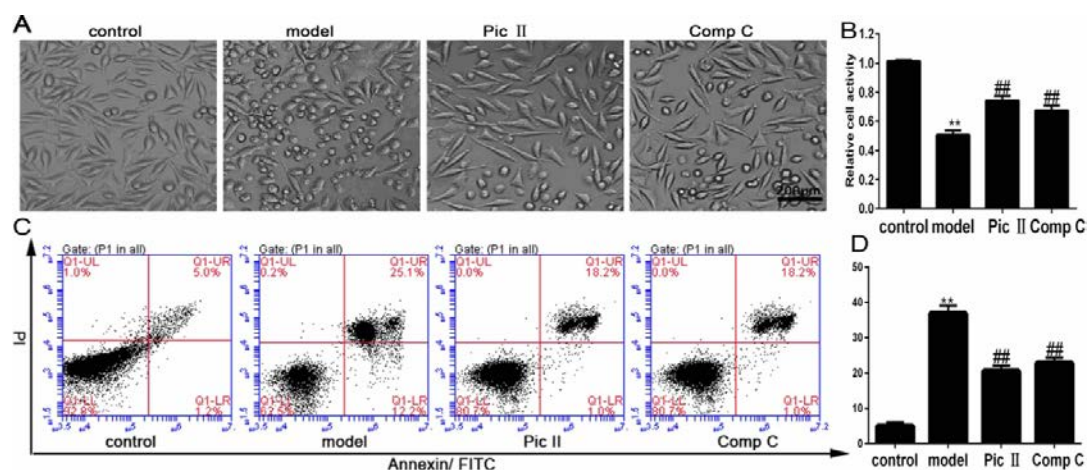
2.9. Statistical Analysis

The SPSS 22.0 software was used for statistical analysis. Measurement data is expressed by mean $\pm$ standard deviation (SD). One-way Anova was used to compare multiple groups. One-way ANOVA and Bonferroni post-special tests were used to determine statistical differences due to normal distribution and equal variance. It is considered that the difference of $P < 0.05$ is statistically significant.

3. Results

3.1. Picoside II alleviated nerve injury in SH-SY5Y cells after OGD/R

As shown in (Figure 1A), the cells grew very well, most of them were long fusiform, triangular or polygonal, with prominent cell protrusions and good adherence in control group. In the model group, most of the cell gaps were enlarged, some cells were irregular in shape, wrinkled in volume, their protuberances were not obvious or even disappeared, and some cells were suspended from the wall in culture medium, and picoside II could reduce cell morphology changes. Specific performance the cell damage was significantly improved, only the cell gap became larger, the synaptic retraction and a small part of the cell deformation, compared with the model group. After compound C treatment, the cell morphology was not significantly different from that of picoside II group.



(A) Morphological changes of cells after OGD/R. Original magnification $\times 100$. Normally growing cells behaved as long fusiform, triangular or polygonal, with prominent cell protrusions and good adherence, while the damaged cells behaved as an increase in cell gap, irregular shape, volume shrinkage, no obvious or disappearing protrusions, and even floating. (B) Viability was determined by CCK-8 assay after OGD/R; (C-D) Flow cytometry analysis of SH-SY5Y cells after OGD/R. Q1-UL (AV-/PI+) represents necrotic cells or mechanically damaged cells; Q1-LL (AV-/PI-) represents living cells; Q1-LR (AV+/PI-) represents early apoptotic cells; Q1-UR (AV+/PI+) represents late apoptotic cells. Representative pictures and quantification for analysis were presented. Data are represented as mean $\pm$ SEM from two independent experiments. * $P < 0.05$, ** $P < 0.01$ vs. control group; # $P < 0.05$, ## $P < 0.01$ vs. model group. Pic II: Picoside II; Comp C: Compound C.

Figure 1. Picoside II alleviated nerve injury in SH-SY5Y cells after OGD/R

It was shown (Figure 1B) that the cell viability of the model group (0.51 ± 0.03) were reduced, compared with the control group (1.01 ± 0.01) ($t=37.53$, $P<0.01$). Treatment with picoside II (0.74 ± 0.04) and compound C (0.67 ± 0.04) could increase cell viability, compared with the control group ($t=10.56$, $P<0.01$) ($t=13.95$, $P<0.01$). There was no significant difference in cell viability between the picoside II group and the compound C group ($F=3.01$, $P>0.05$).

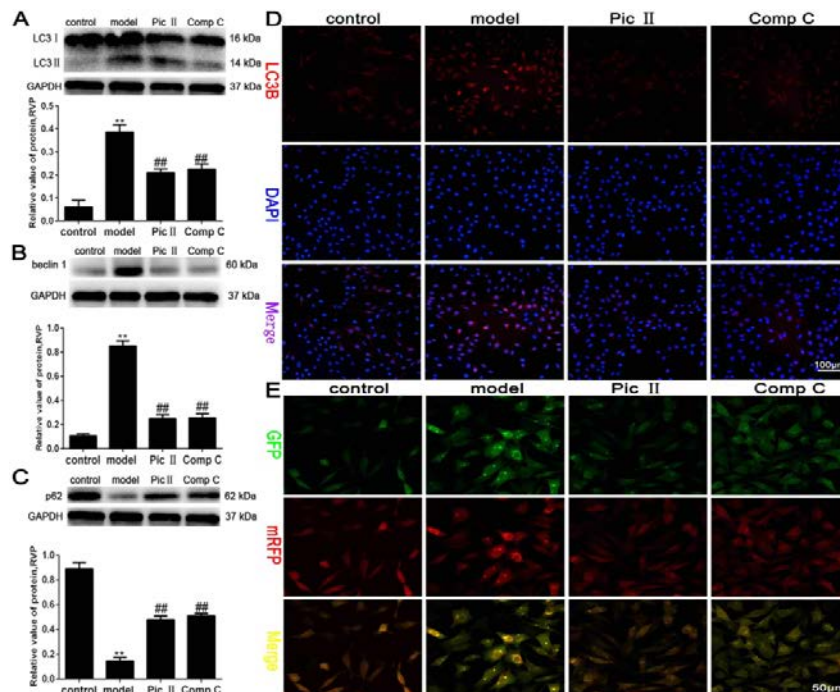
Cells were stained with AV/PI and analyzed (Figure 1C-D) by flow cytometry, and percentages of intact cells (AV-/PI-) and apoptotic cells (AV+/PI-, AV-/PI+ and AV+/PI+) were presented. Compare to control ($4.21 \pm 1.18\%$) group, apoptotic cells increased in model group ($36.90 \pm 2.20\%$) with ($t=37.20$, $P<0.01$), and treatment with picoside II ($20.47 \pm 1.58\%$) and compound C ($22.47 \pm 1.48\%$) could alleviate the apoptosis induced by OGD/R ($t=37.20$, $P<0.01$) ($t=37.20$, $P<0.01$). There was no significant difference in the reduction of apoptosis between the picoside II group and the compound C group ($F=2.94$, $P>0.05$).

3.2. Picoside II Inhibits Autophagy in SH-SY5Y Cells After OGD/R

As shown in (Figure 2A-C), the expression of LC3 (0.06 ± 0.03) and Beclin 1 (0.10 ± 0.02) were low in control group, and p62 levels (0.89 ± 0.09) were high in control group. Compare with control group, the protein levels of LC3 (0.39 ± 0.03) and Beclin 1 (0.85 ± 0.04) increased with ($t=14.08$, $P<0.01$) (51.19 , $P<0.01$) in model group, and the levels of p62 (0.14 ± 0.03) were decreased with ($t=28.05$, $P<0.01$). Treatment with picoside II and compound C

could decrease the expression of LC3 II/LC3 I (0.21 ± 0.02) (0.22 ± 0.02) with ($t=10.94$, $P<0.01$) ($t=36.40$, $P<0.01$), Beclin 1 (0.25 ± 0.03) (0.25 ± 0.04) levels with ($t=16.94$, $P<0.01$) ($t=15.46$, $P<0.01$) and increase the expression of p62 (0.48 ± 0.03) (0.51 ± 0.02) levels with ($t=11.61$, $P<0.01$) ($t=21.57$, $P<0.01$), compare with control group. There was no significant difference in expression of LC3 II/LC3 I, Beclin 1 and p62 levels between the picoside II group and the Compound C group ($F=1.26$, $P>0.05$) ($F=0.44$, $P>0.05$) ($F=1.76$, $P>0.05$). As shown in (Figure 2D), for LC3B was mainly observed by immunofluorescence staining. The expression levels of LC3 II showed low-density fluorescence and observed to diffused in the cytoplasm in control group, while it showed high-density fluorescence and accumulates in the cytoplasm in model group. Cells treat with picoside II and compound C showed low-density fluorescence and reduced cytoplasmic aggregation, compared with the model group.

Autophagy is a dynamic process. In order to track the dynamic process of autophagy formation and degradation, we established stable SH-SY5Y cells that stable expressed a tandem GFP-mRFP-LC3 adenovirus. As shown in (Figure 2E), we could barely see fluorescent dots in control group, while there were a relatively large number of yellow dots (autophagosomes) and red dots (autolysosome) in model group, compare with control group. However, treatment with picoside II and compound C reduced fluorescent spots. The above results suggest that both picoside II and compound C could inhibit autophagy flux in SH-SY5Y cells after OGD/R.



(A-C) Representative immunoblots and quantification for analysis of LC3, Beclin 1 and p62 in SH-SY5Y cells after OGD/R. (D) Immunocytochemical staining of LC3B (red) and DAPI (blue) in SH-SY5Y cells after OGD/R. Original magnification $\times 200$. Red fluorescent labeled positive cells, blue fluorescent labeled total number of nuclei. (E) Picoside II inhibited the formation of autophagosomes and autophagosomes in SH-SY5Y cells after OGD/R. Original magnification $\times 400$. The fluorescence was diffused in the cytoplasm when autophagy was not activated; and the cytoplasm was fluorescently aggregated and forms fluorescent dots, with the activation of autophagy. Yellow dots represent autophagosomes and red dots represent autophagic lysosomes. Data are represented as mean $\pm$ SEM from two independent experiments, * $P < 0.05$, ** $P < 0.01$ vs. control group; # $P < 0.05$, ## $P < 0.01$ vs. model group. Pic II: Picoside II; Comp C: Compound C.

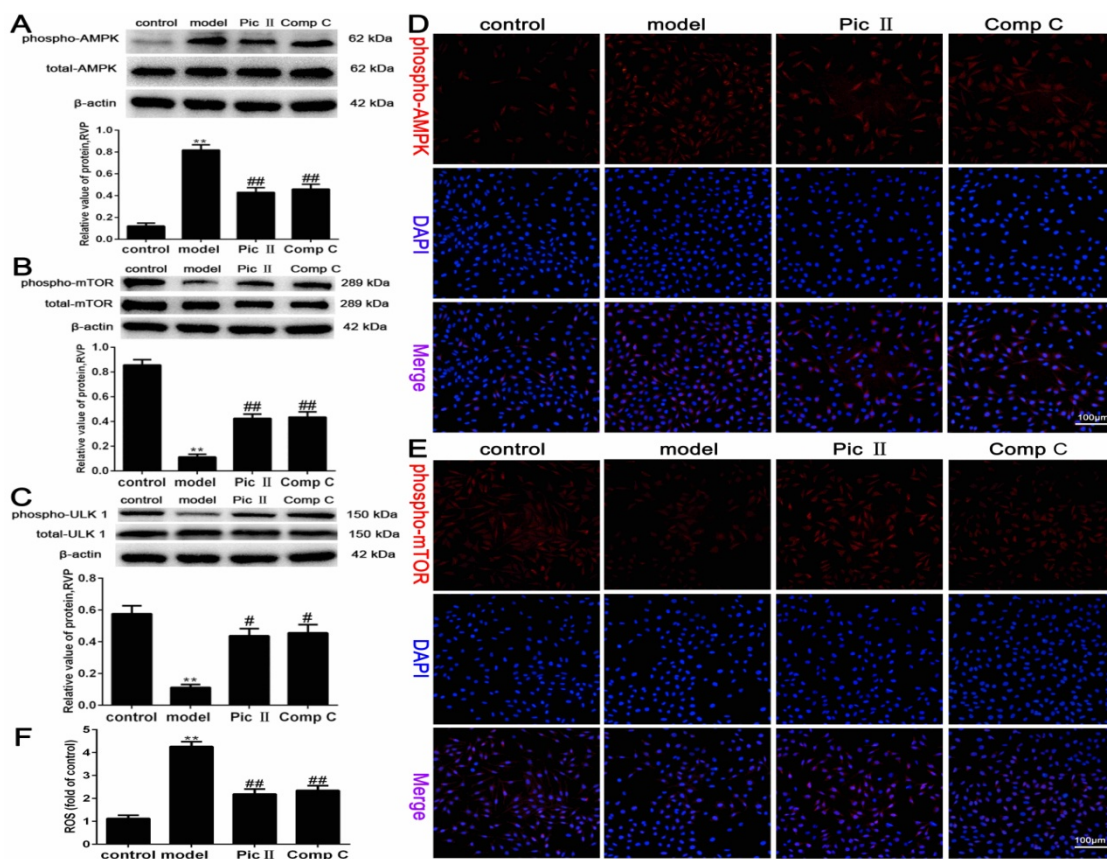
Figure 2. Picoside II inhibits autophagy in SH-SY5Y cells after OGD/R

3.3. Picoside II Inhibited the Activation of AMPK-mTOR-ULK I Signaling Pathway by Inhibiting ROS Production

The Western Blot shown (Figure 3A-C) that the level of phospho-AMPK (0.82 ± 0.05) was increased, while levels of phospho-mTOR (0.11 ± 0.02) and phospho-ULK 1 (0.11 ± 0.02) were decreased, compare with control group ($t=37.01$, $P<0.01$) ($t=49.45$, $P<0.01$) ($t=13.82$, $P<0.01$). Furthermore, treatment with picoside II down-regulated the phospho-AMPK (0.43 ± 0.04) expression and up-regulated the phospho-mTOR (0.42 ± 0.03) and phospho-ULK 1 (0.44 ± 0.05) levels, compare to model group ($t=39.25$, $P<0.01$) ($t=15.69$, $P<0.01$) ($t=10.29$, $P<0.01$). The correlation between the activation of AMPK signaling and autophagy inhibition by picoside II in SH-SY5Y cells after OGD/R was further evaluated using the AMPK-specific inhibitor compound C. Compare with model group, AMPK inhibition up-regulated the phospho-mTOR (0.43 ± 0.04) and phospho-ULK 1 (0.46 ± 0.05) levels with ($t=14.82$, $P<0.01$) ($t=9.89$, $P<0.01$). However, compare with picoside II group, there were no significant difference in expression of phospho-mTOR and phospho-ULK 1 levels ($F=1.68$, $P>0.05$) ($F=3.31$, $P>0.05$). For the phospho-AMPK and phospho-mTOR were mainly observed by immunofluorescence staining. The expression of phospho-AMPK as shown in (Figure 3D), the control

group showed low-density fluorescence and observed in cytoplasm and nucleus, while cells treat with OGD/R showed high-density fluorescence and enriched in cytoplasm. Cells treat with picoside II and compound C showed low-density and reduced aggregation in the cytoplasm, compared with the model group. The expression of phospho-mTOR as shown in (Figure 3E), the control group showed high-density fluorescence, while cells treat with OGD/R showed low-density fluorescence. Cells treat with picoside II and compound C showed high-density, compared with the model group. The expression trends of these two proteins are consistent with the expression of Western blot.

Considering the role of ROS in OGD/R injury and autophagy, we further examined the correlation between ROS and AMPK pathway (Figure 3F). ROS were in a low level in the control group (1.08 ± 0.21). After OGD/R, ROS levels (4.24 ± 0.26) increased significantly, compared with the control group ($t=17.48$, $P<0.01$). Compared with the model group, picoside II treatment down-regulated ROS levels (2.19 ± 0.21) with ($t=13.07$, $P<0.01$), and compound C (2.35 ± 0.20) also inhibited ROS generation with ($t=17.40$, $P<0.01$). There is no significant difference in the inhibition of ROS generation between picoside II and compound C ($F=1.49$, $P>0.05$) in SH-SY5Y cells after OGD/R. Combined with the above results, ROS was involved in AMPK pathway.



(A-C) Representative immunoblots and quantification for analysis of phospho-AMPK, phospho-mTOR and phospho-ULK 1 in SH-SY5Y cells after. (D-E) Immunocytochemical staining of phospho-AMPK and phospho-mTOR (red) and DAPI (blue) in SH-SY5Y cells after OGD/R. Original magnification $\times 400$. Red fluorescent labeled positive cells, blue fluorescent labeled total number of nuclei. Scale bars: 100 μ m. Red fluorescent labeled positive cells, blue fluorescent labeled total number of nuclei. (F) Intracellular reactive oxygen species (ROS) detection. Data are represented as mean $\pm$ SEM from two independent experiments, * $P < 0.05$, ** $P < 0.01$ vs. control group; # $P < 0.05$, ## $P < 0.01$ vs. model group. Pic II: Picoside II; Comp C: Compound C.

Figure 3. Picoside II inhibited the activation of AMPK-mTOR-ULK I signaling pathway by inhibiting ROS production

4. Discussion

Autophagy is an evolutionarily conserved catabolic process by degrading long-lived proteins and damaged organelles in cells [18]. The body can activate autophagy under conditions of starvation, hypoxia, stress, etc [19]. Recent studies have demonstrated that ROS is recognized as one of the fundamental signals for inducing autophagy under stress conditions [20]. Oxidative stress caused by ROS could cause apoptosis of nerve cells [21]. These results suggested that cellular damage caused by oxidative stress may be related to the regulation of autophagy. Autophagic flux refers to the dynamic process of autophagy, which involves the formation of autophagosomes, the transfer of autophagic substrates to lysosomes, and the degradation of autophagic substrates in lysosomes. Autophagy was precisely regulated by autophagy-related genes. Beclin 1, LC3, p62 are mainly autophagy markers. Beclin 1 is essential for the formation of autophagosomes, promoting the transformation of LC3 I to LC3 II and inducing autophagy [22]. Therefore, expression of Beclin 1, LC3, and p62 are usually used to indicate autophagy flux after brain injury. In this present, cerebral ischemia-reperfusion injury can cause the expression of LC3 and Beclin 1 to be up-regulated compared with the sham-operated group and the control group, while the expression of p62 is down-regulated.

Hypoxia/reoxygenation (H/R)-induced cardiomyocyte injury in vitro can be alleviated by inhibiting autophagy flux [23]. Autophagy increased neuronal damage in primary cortical neurons under OGD/R conditions, whereas inhibition of autophagy attenuated neuronal damage after OGD/R [24]. Autophagy was activated after cerebral ischemia reperfusion injury, inhibiting autophagy flux could reduce reperfusion injury [25,26]. Based on above, inhibition of autophagy may be a potential therapeutic strategy to reduce cerebral ischemia reperfusion injury. Simultaneously, we also saw the up-regulation of autophagy flux through the aggregation of GFP-mRFP-LC fluorescent dots after OGD/R of SH-SY5Y cells. Moreover, picoside II treatment markedly down-regulated OGD/R-induced autophagy. Meanwhile, we also observed the effects of picoside II on after OGD/R of SH-SY5Y cells, cell viability, morphological changes, and cell apoptosis rate. After treatment with picoside II significantly improved the cell viability was increased, abnormal cell morphology was significantly improved. These results suggest that picoside II exerts the neuroprotective effect against OGD/R injury via inhibiting autophagy *in vitro*.

To date, the AMPK-mTOR-ULK 1 signaling pathway has been shown to be involved in the process of autophagy [27]. AMPK has been shown to be an important mediator of autophagy induction in glucose withdrawal responses, and essential for cell protection under these conditions [28]. mTOR is a serine/threonine kinase that promotes metabolism and negative feedback regulation autophagy [29]. ULK1, the mammalian homolog of yeast Atg1, is the downstream target of mTOR and one of the hallmarks of autophagy initiation. Under stress of cells, AMPK, an upstream regulator of

mTOR, is activated and followed by phosphorylate mTOR on ser 2448, mTOR is inhibited by phosphorylation TSC complex or phosphorylation of Raptor [30]. Subsequently, phospho-ULK 1 on ser757 is decreased or phospho-ULK 1 on Ser 317 and Ser 777 is activated, which in turn initiates autophagy [30]. In our data, we further explored the mechanism of the neuroprotective effect of picoside II by inhibiting autophagy *in vitro*. The data showed that phospho-AMPK levels were dramatically up-regulated, while phospho-mTOR levels were remarkably down-regulated, subsequently phospho-ULK 1 (ser757) levels were decreased, after SH-SY5Y cells of OGD/R. After treatment with picoside II, the protein level of phospho-AMPK was decreased, the levels of phospho-mTOR and phospho-ULK 1 were increased. Besides, the autophagy fluxes were down-regulated. These results showed that picoside II inhibiting AMPK-mTOR-ULK 1 autophagy signaling pathway after OGD/R.

To further investigate whether picoside II inhibited autophagy was AMPK dependent, we inhibited AMPK activity by compound C, an AMPK inhibitor. The result showed that compound C down-regulated of phospho-AMPK and phospho-ULK 1, and up-regulated of phospho-mTOR expression. In addition, compound C reduced autophagy flux, which was verified by down-regulated the Beclin 1, LC3 II expression and GFP-mRFP-LC3 dots, and up-regulated the p62 expression. Besides, Compound C treatment reduced the damage of SH-SY5Y cells after OGD/R. These results were not significantly different from results of treatment with picoside II. Based on those, compound C and picoside II not only had a certain synergistic effect in reduced autophagy flux, but also there was obvious synergistic effect on the AMPK-mTOR-ULK 1 signaling pathway. Picoside II exhibited a neuroprotective effect through inhibiting AMPK-mTOR-ULK 1 autophagy signaling pathway.

In our data, flow cytometry showed that after OGD/R of cells can induce cell apoptosis. Besides, ROS detection indicated that OGD/R could induce the generation of ROS. Many kinases, such as mitogen-activated protein kinases (MAPK) and AMPK can be activated by ROS [30], while AMPK plays an important role in cellular survival through regulating metabolic balance [16]. In this present, picoside II inhibited the generation of ROS and significantly protected SH-SY5Y cells from OGD/R injury. In addition, inhibition of ROS by picoside II weakened ROS-mediated activation of AMPK and the expression of autophagy flux, indicating that the role of ROS in AMPK activation and subsequent autophagy regulation. These results confirmed that picoside II inhibited ROS production after OGD/R, and down-regulated ROS prevented the activation of AMPK and further inhibited autophagy. In conclusion, the present study showed picoside II can exert neuroprotective effects by inhibiting autophagy in OGD/R injury *in vitro*. This effect of picoside II may be related to inhibition of ROS-mediated AMPK-mTOR-ULK 1 autophagy signaling pathway.

Founding

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Ethics Approval and Consent to Participate

All related experiments were approved by the Ethics Committee of Integrative Medicine Institute, Qingdao University Medical College (QDYXB-WZLL20220623).

Conflicts of Interest Statement

The authors declare that there are no conflict of interest.

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